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Restorative Effect of *Asparagus racemosus* on Age Related Oxidative Damage in Heart Lysosome of Aged Rats

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Abstract: In the present study, we have evaluated the salubrious role of asparagus racemosus root extract (ARRE) on accumulation of oxidative damage products such malondialdehyde (MDA), protein carbonyls (PCO), lysosomal marker enzymes acid phosphatase and cathepsin D activity, aging marker lipofuscin and membrane bound H^+ ATPase activity in heart lysosome of aged rats. Male albino rats of Wistar strain were divided into four groups: Group 1, young control rats; Group 2, young rats treated with ARRE (500 mg kg^{-1} b.wt.) for four weeks; Group 3, aged control rats; Group 4, aged rats supplemented with ARRE (500 mg kg^{-1} b.wt.) for four weeks. Present results, thus, revealed that ARRE has inhibiting effect on the accumulation of age-related oxidative damages and restored the enzyme activity and decreased the lipofuscin content in heart lysosomes. This restorative activity of ARRE mainly attributed to the presence of enriched therapeutic phytochemical constituents, which act synergistically to alleviate the indices of oxidative stress, associated with aging.

Key words: *Asparagus racemosus* root extract, lysosome, aging, lipofuscin, malondialdehyde, protein carbonyl

INTRODUCTION

Aging is characterized by slow, progressive, structural and functional changes that take place at cellular, tissue and organ level. These changes resulting in gradual functional decline, decreased adaptability and ability to face stress and increased probability of age associated diseases including cardio vascular disease, cancer, diabetic, Alzheimer's etc. Age associated changes occur in many cellular organelles including lysosomes and mitochondria. Lysosomes of heart show the most remarkable age-related changes because they are non-proliferative which are played a major role in cellular aging and death. Cellular aging represents slowly developing functional decline of lysosomes compartment and secondary to oxidant-induced damage and lipofuscin accumulation (Terman *et al*., 2006).

Lysosomes are membrane bound vesicles involved in intracellular digestion. They contain a variety of hydrolytic enzymes that are optimally active at an acidic pH. (Holtzman, 1989; Kornfeld and Mellman, 1989; Futai *et al*., 1998). These hydrolases require an acidic environment for activity and become inactivated at a neutral pH. The intralysosomal environment is maintained at pH 4.5 by membrane integrated H^+ ATPase. (Dell'Angelica *et al*., 2000). Lysosomal enzymes degrade not only cytosolic biomolecule like protein, carbohydrate, lipid and nucleic acid but also whole organelles including mitochondria, endoplasmic reticulum, ribosome, peroxisome and proteosomes (Cuervo, 2004; Levine and Klipnsky, 2004). Normally damaged macromolecules and organelles are efficiently degraded in the lysosoome, resulting in the successful recycling (Brunk and Terman, 2002).

In age advances, lysosomal degradation capacity decreases that contribute to increased accumulation of incompletely degraded intralysosomal waste material as lipofuscin (age pigment) which starts to accumulate in postmitotic cells from early life and then gradually increases (Terman *et al*., 2006). Lipofuscin consider as a hall mark of aging and their deposition ultimately decreases cellular adaptability and promotes the development of age-related pathologies, including neuro degenerative diseases, heart failure and macular degeneration (Terman and Brunk, 2004).

The enhancement of lipofuscin formation by oxidative stress and its attenuation by the use of antioxidant and iron chelators suggest a potential anti-aging strategy (Terman *et al*., 2006). The substance centrophenoxine is claimed to promote the removal of lipofuscin from cells, which is consistent with the antioxidant properties (Nagy *et al*., 1994). Plant and plant products are being used as a source of medicine since long. The medicinal properties of

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plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, low toxicity and economic viability (Auddy *et al*., 2003).

Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic etc., (Miller, 1996). They were also suggested a potential iron chelator (Boyer *et al*., 1988; Havsteen, 1983 and Borsari *et al*., 2001). Plant extracts increasingly used as phytotherapeutics and are still a large source of natural antioxidant. Natural antioxidants strengthen the endogenous antioxidant defense from ROS ravage and restore the optimal balance by neutralizing the reactive species. They are gaining immense importance by virtue of their critical role in healthy old age and disease prevention.

In Indian system of medicine *Asparagus racemosus* Willd root (Liliaceae) (Eng: Willd asparagus, Tamil: Thanner Vittan Kizhangu,) is an important medicinal plant. Traditionally it is used as health tonic (Pandey and Chunekar, 1998) and common Indian home remedy used as a rejuvenator, promoter of strength, breast milk and semen (Dash, 1991). Roots of the plant have been used in the Indian traditional system of medicine for the treatment of various ailments in human being (Kirtikar and Basu, 1975; Nadkarni, 1976; Goyal *et al*., 2003). *Asparagus racemosus* is a well known ayurvedic rasayana which prevent aging, increase longevity, impart immunity, improve mental function and add vigor and add vitality to the body and also used in nervous disorders, dyspepsia, tumors, inflammation, hyperdipsia, neuropathy and hepatopathy (Sharma, 2001). *Asparagus racemosus* has also been reported to have potent adaptogenic activity (Rege *et al*., 1999) and antioxidant property (Kamat *et al*., 2000).

The aim of this study was to evaluate the salubrious role of asparagus racemosus root extract (ARRE) on accumulation of oxidative damage products such malondialdehyde (MDA), Protein carbonyls, lysosomal marker enzymes such acid phosphatase and cathepsin D activity, aging marker lipofuscin and membrane bound H^+ ATPase activity in heart lysosome of aged rats.

MATERIALS AND METHODS

Plant material: The roots of the *Asparagus racemosus* were collected from the kolli hills, Tamil Nadu, South India. The collected roots were identified and authenticated by a botanist Prof. Dr. M. Jegadeesan, Department of Environmental and Herbal Science, Tamil University, Thanjavur, Tamil Nadu. A Voucher specimen (Specimen No. 29) has been deposited at the Herbarium of our department. The roots were cut into small pieces and shade dried at room temperature for 15 days and powdered finely then used for extraction.

Preparation of plant extract: A required quantity of the powder (5 g) was suspended in a measured amount of distilled water (600 mL). The suspension was boiled until the quantity was reduced to 100 mL. The resultant decoction was cooled and used in the present study. The concentration of resultant decoction was 50 mg mL^{-1}. For experiments 500 mg kg^{-1} body weight of asparagus racemosus root extract (ARRE) was used. This effective dose of ARRE was selected based on the dose dependent studies carried out in aged rats (Velavan *et al*., 2006).

Animal: Male albino rats of wistar strain approximately 3-4 months old rats weighing approximately 140-160 g (young) and 24-26 months old rats weighing approximately 380-410 g (aged) were used in this study. They were healthy animals from Sri Venkateswara enterprises, Bangalore, India. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (Temperature 27±2°C and 12 h light/dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet (Gold Mohur, Mumbai, India) and water *ad libitum*. They were acclimatized to the environment for 1 week prior to experimental use. The study protocol was carried out as per the rules and regulation of the Institutional Animal's Ethics Committee (IAEC).

Chemicals: Eethylene Diamine Tetra Acetic Acid (EDTA), Trichloro Acetic Acid (TCA), Thio Barbituric Acid (TBA), Tris-HCL and Guanidine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade with high purity and were obtained from Glaxo Laboratories, Mumbai, India and Sisco Research Laboratories, Mumbai, India.

Grouping of animals: Body weights of the animals were recorded and they were divided into 4 groups of 6 animals each as follows.

Group 1: Control young rats.
Group 2: Young rats administered ARRE (500 mg kg^{-1} b.wt day^{-1}) orally for 4 weeks.
Group 3: Control aged rats.
Group 4: Aged rats administered ARRE (500 mg kg^{-1} b.wt day^{-1}) orally for 4 weeks.

After the completion of experimental regimen, the rats were fasted over night. The heart was dissected out and washed in ice-cold saline and weighed. A known weight of them was used for homogenate preparation. Lysosomes (pellet) were isolated from the homogenate and used for various biochemical analyses. Lysosomal marker enzymes as acid phospatase and cathepsin D were measured in both the post-lysosomal supernatant (PMS) and the pellet.

Isolation of lysosomes: Heart lysosomes were isolated by the method of Allan and Welman (1980) with minor modification. A 10% tissue homogenate was prepared in 0.25 M sucrose/20 mM-Tris/HCl (pH 7.8). According to Ignarro (1971), this buffer helps to maintain the integrity of lysosomes during homogenization. The homogenate was centrifuged at 600 g for 10 min at 4°C. The pellet (nuclear fraction) was discarded and post-nucleat supernatant was recentrifuged at 9000 g for 15 min at 4°C. The pellet (mitochondrial fraction) was removed and Post-mitochodrial supernatant was recentrifuged at 16,000 g for 30 min at 4°C to obtain the lysosomal enriched pellet. Lysosomal pellet was suspended in 5 mL of homogenizing medium. Lysosomal protein was estimated by the method of Lowry *et al.* (1951).

Determination of MDA and PCO: Protein carbonyls content was determined by the reliable method based on the reaction of carbonyl groups with 2,4-dinitro-phenylhydrazine to form 2,4-dinitro-phenylhydrazone as suggested by Levine *et al.* (1994). The protein carbonyl content was expressed as nmol of DNPH incorporated mg^{-1} protein in tissues. Malondialdehyde (MDA) was estimated by the thiobarbituric acid assay method of Beuge and Aust (1978). The results were expressed as nmoles MDA formed mg^{-1} protein in tissues.

Determination of acid phosphatase and cathepsin-D in post lysosomal supernatant: Acid phosphatase activity was measured by the method of Annon (1963). The acid phosphatase activity was expressed as µmol of phenol liberated min^{-1} mg^{-1} protein. Cathepsin-D was measured by the method of Takahashi and Tang (1981). Cathepsin-D is expressed µmol of tyrosine liberated h^{-1} mg^{-1} protein.

Determination of lipofuscin and H^+ATPase activity: Lipofuscin concentration measured by the method of Tappel *et al.* (1973). Lipofuscin content was expressed as fluorescence arbitrary units gm^{-1} tissue. H^+ATPase assay was assayed according to the protocol Gupta *et al.* (1991) and Manzoor *et al.* (1999) with some modifications. The H^+ATPase activity was assessed by the measurement of the produced inorganic phospate by Fiske and Subbarrow (1925) method and results were expressed as specific activity (µmoles of inorganic phosphorus librated h^{-1} mg^{-1} protein).

Statistical analysis: All the values were expressed as means±SD of six rats from each group and statistically evaluated by one-way analysis of variance (ANOVA). The means were tested for significance by Tukey's test for multiple comparisons (Harvey and Searle, 1998). A value of $p<0.001$ was considered as significant.

RESULTS

The Group 3 aged rats showed a significant increase in MDA, PCO and lipofuscin in heart lysosome as compared to Group 1 control young rats (Table 1). ARRE treated Group 4 aged rats showed a significant decrease in MDA, PCO and lipofuscin in heart lysosome as compared to Group 3. The Group 3 aged rats showed a significant increases in cathepsin D, acid phasphatase and significant decrease of H^+ATPase activity in heart lysosome as compared to Group 1 control young rats (Table 2). ARRE treated Group 4 aged rats showed a significant decrease in cathepsin D, acid phasphatase activity and a significant increase H^+ATPase activity in

Table 1: Effect of *asparagus racemosus* on MDA, PCO and lipofuscin in heart lysosome of control and experimental animals

	Young rats		Aged rats	
Parameters	Group 1 (control)	Group 2 (ARRE treated)	Group 3 (control)	Group 4 (ARRE treated)
MDA (U*)	1.69±0.12	1.49±0.09	2.49±0.16[a]	1.83±0.13[bNS]
PCO (U**)	4.54±0.26	4.34±0.23	5.34±0.23[a]	4.71±0.22[bNS]
Lipofuscin (U***)	6.84±0.218	6.58±0.210	8.19±0.262[a]	7.03±0.224[bNS]

U* nmoles of MDA formed/mg protein, U** nmol of DNPH incorporated/mg protein, U*** fluorescence arbitrary units/gm tissue, [a] as compared with group 1 young control rats ($p<0.001$), [b]as compared with group 3 aged control rats ($p<0.001$), [NS]as compared with group 1young control rats (Non-significant)

Table 2: Changes in activities of cathepsin D, acid phasphatase and H^+ATPase in heart lysosome of control and experimental animals

	Young rats		Aged rats	
Parameters	Group 1 (control)	Group 2 (ARRE treated)	Group 3 (control)	Group 4 (ARRE treated)
Cathepsin D (U*)	32.65±2.00	34.78±1.12	41.32±1.09[a]	35.86±3.12[bNS]
Acid phospatase (U**)	8.84±0.58	9.27±0.67	12.49±1.27[a]	10.01±1.02[bNS]
H^+ ATPase (U***)	4.23±0.030	4.26±0.042	3.76±0.025[a]	4.19±0.036[bNS]

U* µmol of tyrosine liberated/hour/mg protein, U** µmol of phenol liberated/min/mg protein, U*** µmoles of inorganic phosphorus librated/ hour/mg protein, [a]as compared with group 1 young control rats ($p<0.001$) [b]as compared with group 3 aged control rats ($p<0.001$), [NS] as compared with group 1young control rats (Non-significant)

heart lysosome as compared to Group 3. In young rats Group 2 ARRE administration showed a lowered MDA, lipofuscin, PCO content and non-significant changes (NS) in all other parameters as compared to Group 1 control young rats.

DISCUSSION

Aging affects all types of muscle cells, yet the degree of age related changes is the highest in cardiac myocytes (Coleman *et al*., 1987). This accords well with differences in oxygen consumption and consequent ROS-induced damage, which is maximal in the heart, as well as with regenerative potential of different muscle tissues, being poorest for myocardium. The cardiac tissue is expected to suffer the maximum damage because it has been shown that post mitotic tissues accumulate damage faster than mitotically active tissues (Kowald and Kirkwood, 2000). Thus, maintenance of lysosomal function may be important to maintain overall myocardial function.

Lysosomes, ubiquitous in all animal cells as an acidic compartment with limiting membranes are able to degrade unneeded intra- and extracellular materials to biological monomers and contain various types of proteinases expect for metalloproteinases (Terman *et al*., 2006). Lipofuscin or age pigment accumulates in the lysosomal vacuome of a variety of post mitotic cell types including heart, during aging in man and animals (Marzabadi and Jones, 1992). Lipofuscin consider as a hallmark of aging, which is primarily composed of oxidatively modified protein and lipid degradation residues. It also contains some carbohydrate and traces of metals including copper, manganese and aluminum. Zinc, calcium and high content of iron (Terman *et al*., 2006). The increased amount of iron within lipofuscin granules, promoting generation of ROS by stimulating Fenton-like reactions. Cellular injury increases due to a rise in ROS generation results in lysosomal membrane destabilization (Essner and Novikoff, 1960; Brunk and Ericsson, 1972), resulting in hydrolytic enzymes leak into the cytosol and trigger apoptotic pathway (Bidere *et al*., 2003; Brunk *et al*., 2001; Cirman *et al*., 2004; Johansson *et al*., 2003; Zhao *et al*., 2003). Thus, these remarkable organelles are primarily meant for the digestion of a variety of biological material and secondarily cause aging and death of animal cells.

The study of lipid peroxidation is attracting much attention in recent years due to its role in disease processes. It has been implicated in the pathogenesis of a number of diseases including aging (Devasagayam *et al*., 2003). It is now generally accepted that lipidperoxidation and its products (MDA) play an important role in liver, kidney, heart and brain toxicity (Poli *et al*., 1987; Cojocel *et al*., 1984; Usyal *et al*., 1989). MDA is the major reactive aldehyde resulting from the peroxidation of biological membrane PUFA and used as an indicator of tissue damage and oxidative stress (Halliwell, 1991; Ohkawa *et al*., 1979; Vaca *et al*., 1988). The occurrence of lipidperoxidation in biological membranes results in impaired membrane function, decreased fluidity, inactiviation of membrane bound receptors and enzymes (Halliwell and Gutteridge, 1989).

Oxidative modification alters the function of proteins and is thought to play an important role in the decline of cellular function during aging. Free radicals produced during oxidative stress can damage the peptide backbone, resulting in the generation of protein carbonyls (Levine and Stadtman, 2001). Lipid peroxidation may also bring about protein damage by inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products, malondialdehyde and 4-hydroxynonenal (Halliwell and Gutteridge, 1999).

The action of reactive oxygen species on lipids and protein increases with age that promote lipidperoxidations and protein oxidation, which are an important key factors in the damage caused to the lysosomal membrane and inactivation of membrane bound ATPase. The level of these oxidized molecules can be quantitated by measurement of MDA and PCO content, which has been shown to increase during aging (Navarro, 2004). Determination of MDA and PCO content used as a marker for membrane damage (Sohal, 2002 and Spiteller, 2001). In the present study, we also observed the increased content of MDA and PCO in heart of aged untreated rats as compared to young rats. The increased content of MDA and PCO indicates the membrane damage in the lysosome. Supplementation of ARRE decreased the content of MDA and PCO in aged rats as compared to young control rats indicate the antioxidative activity of ARRE. Our earlier reports reveals that the quantitative analysis of ARRE indicated the presence of flavonoids (36.7±3.9 mg 100 mL^{-1}), polyohenols (88.2±9.3 mg 100 mL^{-1}) and vitamin-C (42.4±5.1 mg 100 mL^{-1}) (Velavan *et al*., 2007). It is well known that the flavonoid and polyphenols are natural antioxidant but also have been reported to significantly decreased MDA and PCO content (Russo *et al*., 2000; Ferguson, 2001; Erlejman *et al*., 2004).

An acidic internal pH is an intrinsic property of lysosomes. The pH of lysosome is maintaining by vacuolar H^+-ATPases (i.e., acidification of the compartment) and provides an optimal lysosomal enzyme function. One of the most important targets of Reactive Oxygen Species (ROS) is the membrane lipid, which undergo peroxidation. Lipid peroxidations are key factors in the damage caused to the lysosomal membrane and

disturb the H^+-ATPases which leads to change in the intralysosomal pH (Carsten, 2004). Impaired lysosomal acidification would be inactivation of lysosomal hydrolases with subsequent introlysosomal storage of undigested material and promotion of lipofuscin formation (Ivy *et al*., 1984). Lipofuscin is considered to be the end product of peroxidation, fragmentation and polymerization of lipids and proteins (Marzabadi and Jones, 1992).

In the present study, decline the activity of H^+-ATPases and increased content of lipofuscin were observed in heart of aged untreated rats when compared to young rats. Reversion of H^+-ATPases activity and decreased levels of lipofuscin in *Asparagus racemosus* treated animals may be due to the stabilizing property of the ARRE on lysosomal membrane which could have been imparted by the flavonoids. The drug may modify the lysosomal membrane in such a way that it is capable of fusing with the plasma membrane and thereby preventing the discharge of acid hydrolases or by inhibiting the release of lysosomal enzymes (Carevic and Djokic, 1988). The stabilizing property of the ARRE on lysosomal membrane to maintain the intralysosomal pH. Acidic nature of lysosomal compartment, promote the activity of hydrolytic enzymes that degraded the lipofuscin. The membrane stabilizing property of ARRE may be due to the presence of flavonoid, polyphenol and vitamin-C. Numerous reports have appeared on the inhibition of release of acid hydrolases by flavonoids. (Havsteen, 1983).

Lysosome contains a battery of hydrolytic enzymes such as acid phosphatase, cathepsin, β glucuronidase etc. If the lysosomal membrane is damaged or destabilized then these marker enzymes are released. Hence the assay of these enzymes can be used as an index of lysosomal membrane damage. The release of lysosomal enzymes is related to necrosis of death of the cell or pathological stress full conditions. Lysosomal damage is well established as a biomarker of stress in a wide range of vertebrates and invertebrates (Bindu *et al*., 2005). The most powerful hydrolytic enzymes are the cathepsins. Cathepsin D (cat D) is a lysosomal aspartic protease that is widely distributed in tissue cells and has been shown to be involved in aging and certain pathological condition (Shibata *et al*., 2001). Acid phosphatase is localized in cellular lysosomes. The digestive enzymes of cellular compounds are confined to the lysosomes in the best interest of the cell. Escape of these enzymes into cytosol will destroy the functional macromolecules of the cell and result in many complications. The occurrence of several diseases (e.g., arthritis, aging, muscle diseases etc.,) has been mainly attributed to the release of lysosomal enzymes (Holtzman, 1989).

In the present study, the significant elevation of acid phosphatase and cathepsin D activity were observed in heart of aged untreated rats as compared to young rats. Results data clearly indicates that aged untreated rats exposed to sever oxidative stress, which leads to lysosomal membrane destabilization. This may be due to aged rats increased accumulation of lipofuscin that causes lipid peroxidation of lysosomal membrane through Fentone like reaction and thereby release of acid phosphatase and cathepsin D into cytosol. Administration of ARRE to aged rats restored the activity of acid phosphatase and cathepsin D in heart indicate that the stabilization of lysosomal membrane and prevent the membrane peroxidation. Present findings is in agreement with the Samarth *et al*. (2001) studies.

CONCLUSIONS

The results of the present study indicate that the restorative effect of ARRE in aged rats may probably related to a counteraction of free radicals by its antioxidant nature of ARRE, to a strengthening of heart lysosomal membrane by its membrane stabilizing action through its ability to decrease the levels of lipofuscin, lipid peroxidation and protein carbonyl which is used as a marker for oxidative damage. This restorative activity of ARRE mainly attributed to the presence of enriched therapeutic phytochemical constituents, which act synergistically to alleviate the indices of oxidative stress, associated with aging.

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